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Cytogenetic characterization and B chromosome diversity in direct-developing frogs of the genus *Oreobates* (Brachycephaloidea, Craugastoridae)

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Abstract

Oreobates Jiménez de la Espada, 1872 is a large group of South American frogs with terrestrial reproduction and direct development, located in the superfamily Brachycephaloidea. About 260 brachycephaloidean species have been cytogenetically studied so far, at least with standard techniques. However, this information represents fewer than 17% species of the family Craugastoridae Hedges, Duellman & Heinicke, 2008, where the genus *Oreobates* is included. In the present work, using a diversity of standard and molecular techniques, we describe the karyotype of *O. barituensis* Vaira & Ferrari, 2008, *O. berdemenos* Pereyra, Cardozo, Baldo & Baldo, 2014 and *O. discoidalis* (Peracca, 1895), from northwestern Argentina. The three species analyzed showed a diploid karyotype with 2n = 22 biarmed chromosomes, fundamental number (FN) = 44, nucleolus organizer regions (NORs) located pericentromerically on pair 7, and a centromeric and pericentromeric C-banding pattern. We observed variations in the chromosome number in *O. barituensis* due the presence of two morphs of B chromosomes, one medium-sized telocentric (B_T) and another subtelocentric and smaller (B_{st}). Both B chromosomes are mitotically

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stable and were recorded in all somatic and germinal cells analyzed. The B_T chromosome occurred at a maximum of one per individual ($2n = 22+B_T$), and the other one was observed single ($2n = 22 + B_{st}$) or as a pair in two doses ($2n = 22 + 2B_T$). We additionally observed other supernumerary chromosomes in the three species analyzed, all of them euchromatic, small, dot-shaped and with instability during mitoses, showing a frequency of occurrence below 50% in studied specimens. The occurrence of polymorphic and spontaneous chromosomal rearrangements and supernumerary chromosomes is a recurrent feature reported in frogs with terrestrial habits (Brachycephaloidea and Hemiphractidae Peters, 1862), which suggests that Brachycephaloidea may be a promising group for studying the origin and maintenance of B chromosomes in anurans.

Keywords

Cytogenetics, accessory elements, ribosomal DNA, Anura

Introduction

Superfamily Brachycephaloidea includes a large group of frogs with terrestrial reproduction and direct development, with more than a thousand species assigned to three families: Brachycephalidae Günther, 1858, Craugastoridae, and Eleutherodactylidae Lutz, 1954 (Frost 2015). From a cytogenetic perspective, about 26% of brachycephaloidean species were studied at least with conventional staining techniques, including 13% of Brachycephalidae (7 spp.), 17% of Craugastoridae (128 spp.) and 57% of Eleutherodactylidae (120 spp.) (Campos and Kasahara 2006, Schmid et al. 2010, Díaz et al. 2012, Kaiser et al. 2015). Brachycephaloidea presents an important karyotypic diversity, with diploid numbers (2n) ranging from 16 to 38, an unusually high frequency of spontaneous and polymorphic Robertsonian rearrangements, B chromosomes, and the occurrence of spontaneous somatic supernumerary chromosomes which do not form polymorphisms (Schmid et al. 2010).

Within Craugastoridae (subfamily Holoadeninae Hedges, Duellman & Heinicke, 2008), *Oreobates* Jiménez de la Espada, 1872 is a South American genus with 23 species that occurs on the lower slopes of the Andes from the upper Amazon basin in southern Colombia to northern Argentina, reaching eastwards some areas in western Brazil (Frost 2015). In Argentina, the genus is represented by three species, *O. berdemenos* Pereyra, Cardozo, Baldo & Baldo, 2014, which is phylogenetically related to *O. lundbergi* and characterized by an incomplete discoidal fold (Pereyra et al. 2014); and the cryptic species, *O. barituensis* Vaira & Ferrari, 2008 and *O. discoidalis* (Peracca, 1895), recovered as sister species in the most inclusive phylogenetic analyses, both with a complete discoidal fold (Padial et al. 2014, Pereyra et al. 2014). When compared to other Brachycephaloidea genera, the cytogenetic information available for *Oreobates* is extremely scarce and only two species were studied to date, *O. discoidalis* (Brum-Zorilla and Sáez 1968 [as *Eleutherodactylus*], Schmid et al. 2010), and *O. crepitans* (Bokermann, 1965) (Siqueira et al. 2009, as [*Pristimantis*]). Both taxa share the same chromosome formulae ($2n = 22$, $FN = 44$) and similar chromosomal morphology. In the latter the heterochromatin pattern and nucleolus organizer regions were described

by C- banding and silver staining (Ag-NORs) respectively, whereas the karyotype of *O. discoidalis* was only studied with standard staining techniques (Giemsa).

B chromosomes (Bs) are dispensable extra chromosomes in the standard karyotype (As) present in many taxa, and usually lack phenotypic effects on their hosts. Their prevalence in animal populations is highly variable, being one of the main causes of chromosomal polymorphism in eukaryotes (Jones and Rees 1982, Beukeboom 1994, Camacho et al. 2000). Until now, the presence of B chromosomes in Brachycephaloidea has been described in three species, *Craugastor* sp., *Eleutherodactylus gundlachi* Schmidt, 1920 and *Oreobates discoidalis*. However, another sort of chromosomal variation due to supernumeraries is frequently observed. Although they are associated with spontaneous chromosomal aberrations, their main difference with Bs lies in the fact that they are not polymorphic (Schmid et al. 2010).

In order to complement the karyotypic information available for *Oreobates*, in the present work we studied three species (*O. barituensis*, *O. berdemenos,* and *O. discoidalis*), from several localities in northwestern Argentina. Chromosome morphology, heterochromatin distribution and composition, and location of nucleolar organizer regions are described. We discuss and evaluate the apparent chromosomal homogeneity observed for the genus *Oreobates,* in contrast to the variability reported by the presence of supernumerary chromosomes.

Material and methods

We studied 64 specimens of both sexes of *O. barituensis* (N = 40), *O. berdemenos* (N = 14), and *O. discoidalis* (N = 10). Chromosome preparations were obtained from bone marrow, gut epithelium and testes in males (Schmid et al. 2010). Animals were euthanized with 5% lidocaine and fixed in 4% formalin. Vouchers were preserved in 70% ethanol and stored in the herpetological collections of Fundación Miguel Lillo, Tucumán, Argentina (FML, and provisional field numbers MSA), and Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical, Posadas, Misiones, Argentina (LGE, and provisional field numbers MSA). The complete list of specimens analyzed, collection sites, sex, and voucher numbers are detailed in the Supplementary file 1 online. The diploid number (2n) and Fundamental Number (FN) were obtained after counting at least ten cells per specimen. Mitotic and meiotic preparations were stained with a phosphate-buffered Giemsa solution (10%). Heterochromatic regions were identified by C-banding (Sumner 1972). Silver-staining (Howell and Black 1980) and fluorescent in situ hybridization (FISH) with a ribosomal 18S biotinylated probe (Pinkel et al. 1986) were carried out to evidence active nucleolar organizer regions Ag-NORs and the presence of repetitive rDNA, respectively. To study the nucleotide composition of heterochromatin (prevalence of repetitive sequences AT and CG), we used the fluorochromes base specific DAPI (4', 6-diamidino-2-phenylindole) and CMA_3 (Chromomycin A_3), following Schweizer (1976). Karyotypes were arranged in decreasing size, according

the nomenclature of Green and Sessions (1991, 2007). The relative length and centromeric index (CI) of chromosomes were obtained from mitotic metaphases using the software Micromeasure 3.3 (Reeves and Tear 2000).

The advertisement call of *O. discoidalis* remains unknown, and those described by Ferrari and Vaira (2008) and Akmentins (2011) correspond to *O. berdemenos* (Pereyra et al. 2014). To avoid misidentification of specimens, collections of *O. discoidalis* were made near the type locality (San Miguel de Tucumán, Argentina), whereas individuals from Jujuy and Salta Provinces were considered as *O. barituensis,* based on their advertisement calls as described by Vaira and Ferrari (2008) and Akmentins (2011). Specimens of *O. berdemenos* were collected at the type locality of the species (Abra Colorada, Jujuy province, Argentina), and Nogalar de los Toldos (Salta province, Argentina). The identity of *O. berdemenos* specimens was confirmed by morphological and/or acoustic characters (Supplementary file 1 online).

Results

Oreobates barituensis, *O. berdemenos*, and *O. discoidalis* shared diploid karyotypes with 22 bi-armed chromosomes (2n = 22; FN = 44). Pairs 1, 2, 5, 6, 8–11 were metacentric, while 3, 4 and 7 submetacentric (Fig. 1; Table 1). In all specimens analyzed of the three species, the C-banding technique showed a high predominance of centromeric and pericentromeric heterochromatin, as well as an interstitial band on the long arm pairs 6 (Fig. 1D–F). However, this band varied in its staining intensity or even was absent in some metaphases of a given slide. DAPI/CMA_3 fluorochromes staining, evidence DAPI positive marks at centromeric and pericentromeric regions on almost all chromosomes (CMA_3 negative). In the three species, CMA_3 positive marks (DAPI negative) were evident only on pericentromeric position over pair 7, coincident with secondary constriction sites (Fig. 2), Ag-NORs (Fig. 3A–C) and with the hybridization signals of 18S rDNA probe after FISH experiments (Fig. 3D–F).

We found variations in the chromosome number in eight specimens of *Oreobates barituensis* as a consequence of two different B chromosomes (Fig. 1G–J; 3G–J; 4). Both supernumeraries were present in every somatic cell analyzed (mitotically stable). One of them, was a telocentric large-sized chromosome, arbitrarily named herein B_T, which reached a maximum of one per cell (2n = 22 + B_T). It was observed in three specimens from Peña Alta (LGE 6203, 4784–5) and two from Normenta (MSA 176, 180). C–banding revealed that this chromosome has a significant amount of heterochromatin in the whole arm, mainly visible in the centromeric region (Fig. 1I). The heterochromatin was DAPI positive/CMA_3 negative with an interstitial mark DAPI negative/CMA_3 positive (Fig. 3I, J). The B_T chromosome showed terminal active Ag-NOR sites after silver staining (Fig. 3G). In situ hybridization with the ribosomal 18S probe showed a terminal mark on B_T matching with Ag-NORs, but also brightly interstitial rDNA heterochromatin associated with negative Ag-NORs (Fig. 3H). The other supernumerary was a subtelocentric small chromosome, named as B_{st}, found in

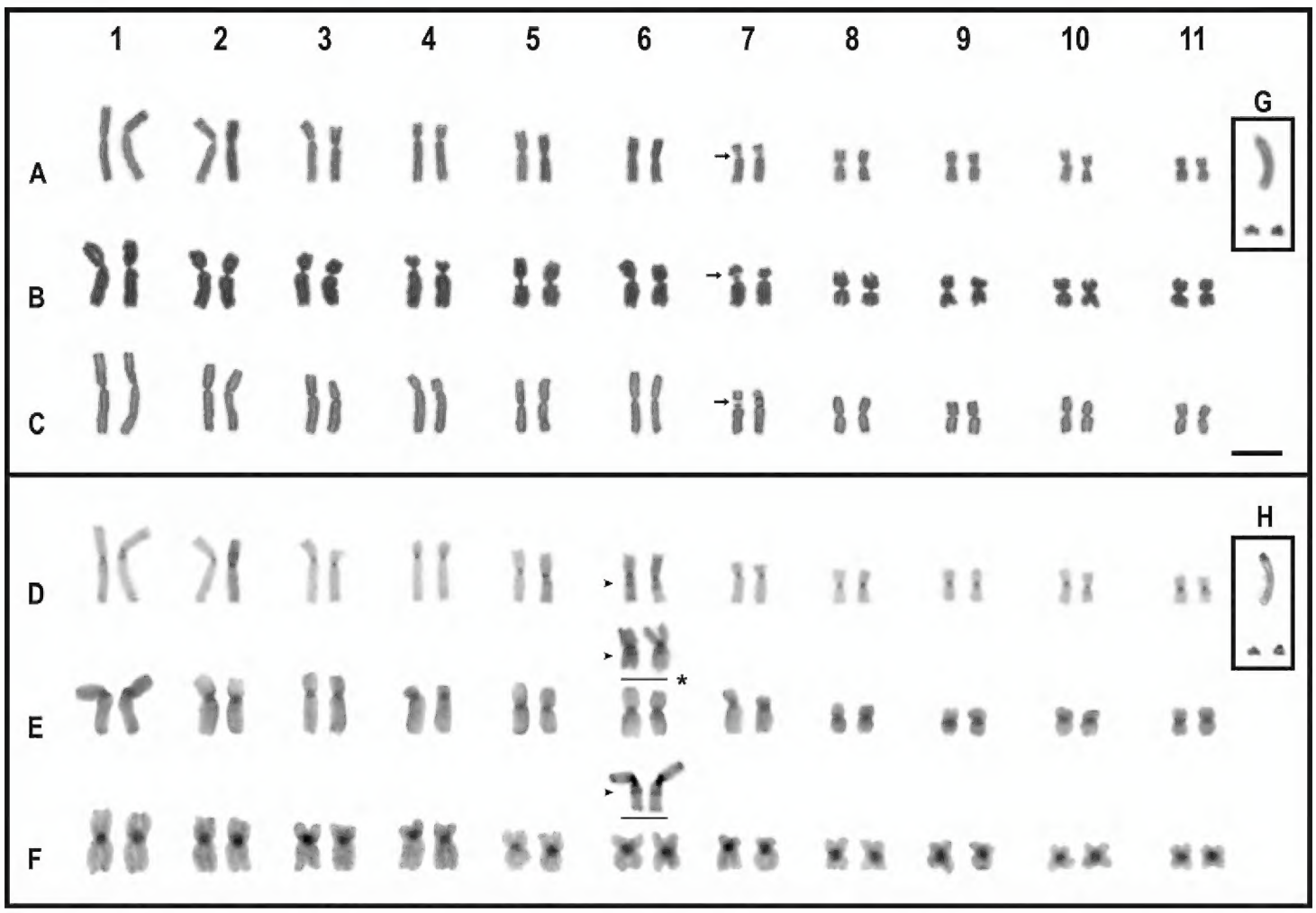

Figure 1. Karyotypes of *Oreobates barituensis* (**A, D**), *O. berdemenos* (**B, E**), *O. discoidalis* (**C, F**), the large telocentric B_T and the small subtelocentric B_{st} supernumerary chromosomes in *O. barituensis*(Boxes **G** and **H**). Giemsa stained (**A–C, G**), C- banding (**D–F, H**). The insets (*) shows interstitial C-bands. Arrowheads point to C positive bands. Bar = 10 μm.

the three specimens analyzed from Tiraxi (MSA 168, 195; LGE 9652). While a single individual carried only one B ($2n = 22 + B_{st}$), the others brought it in a double dose ($2n = 22 + 2B_{st}$). After C-banding, this small sized B_{st} stained darker than A chromosomes, with a conspicuous darker heterochromatic centromere (Fig. 1J), positive for DAPI (Fig. 4A–C).

In addition, eight specimens of *O. barituensis* (LGE 4785; 6202; MSA 127–8, 161, 164, 174, 177), four *O. berdemenos* (FML 24626, MSA 138, 142–3), and one *O. discoidalis* (FML 24513), showed variations in the chromosome number attributable to dot-shaped and mitotically unstable supernumerary chromosomes, named here B_d. These elements share a similar shape with no evident primary constrictions, and no more than one per metaphase was observed (i.e. $2n = 22$; $22 + B_d$), with an occurrence below 50% per individual. C-banding and DAPI staining showed mostly a euchromatic nature of these elements (Fig. 4E).

The Meiosis I analyses on males of *O. barituensis* with supernumerary chromosomes B_T and B_{st} evidenced the presence of 11 bivalents, corresponding to the A standard complement in addition to Bs. The B_T chromosome occurred as a single univalent with not differentiable pyknosis from other chromosomes (Fig. 5A). On the other hand, metaphases I from specimens with B_{st} showed a clearer staining than As (Fig.

Table 1. Chromosome morphology in the three species of *Oreobates*. Chromosome types according to Green and Sessions (1991), Centromeric Index (CI), metacentric (m: 0.500 to 0.375); submetacentric (sm: 0.374 to 0.250); SD = Standard Deviation.

Chromosome number	1	2	3	4	5	6	7	8	9	10	11
Oreobates barituensis											
%Set	15%	12%	11%	11%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.43 ± 0.02	0.38 ± 0.03	0.37 ± 0.05	0.28 ± 0.06	0.45 ± 0.02	0.43 ± 0.03	0.34 ± 0.04	0.46 ± 0.02	0.46 ± 0.02	0.45 ± 0.03	0.47 ± 0.03
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**
Oreobates berdemenos											
%Set	15%	12%	11%	11%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.42 ± 0.02	0.38 ± 0.02	0.35 ± 0.05	0.25 ± 0.04	0.44 ± 0.02	0.45 ± 0.02	0.33 ± 0.04	0.45 ± 0.05	0.47 ± 0.02	0.45 ± 0.03	0.46 ± 0.03
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**
Oreobates discoidalis											
%Set	15%	12%	11%	10%	10%	9%	8%	7%	6%	6%	6%
CI ± SD	0.44 ± 0.02	0.39 ± 0.02	0.37 ± 0.04	0.29 ± 0.04	0.45 ± 0.02	0.44 ± 0.03	0.27 ± 0.05	0.46 ± 0.02	0.46 ± 0.03	0.43 ± 0.05	0.48 ± 0.01
Type	**m**	**m**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**	**m**

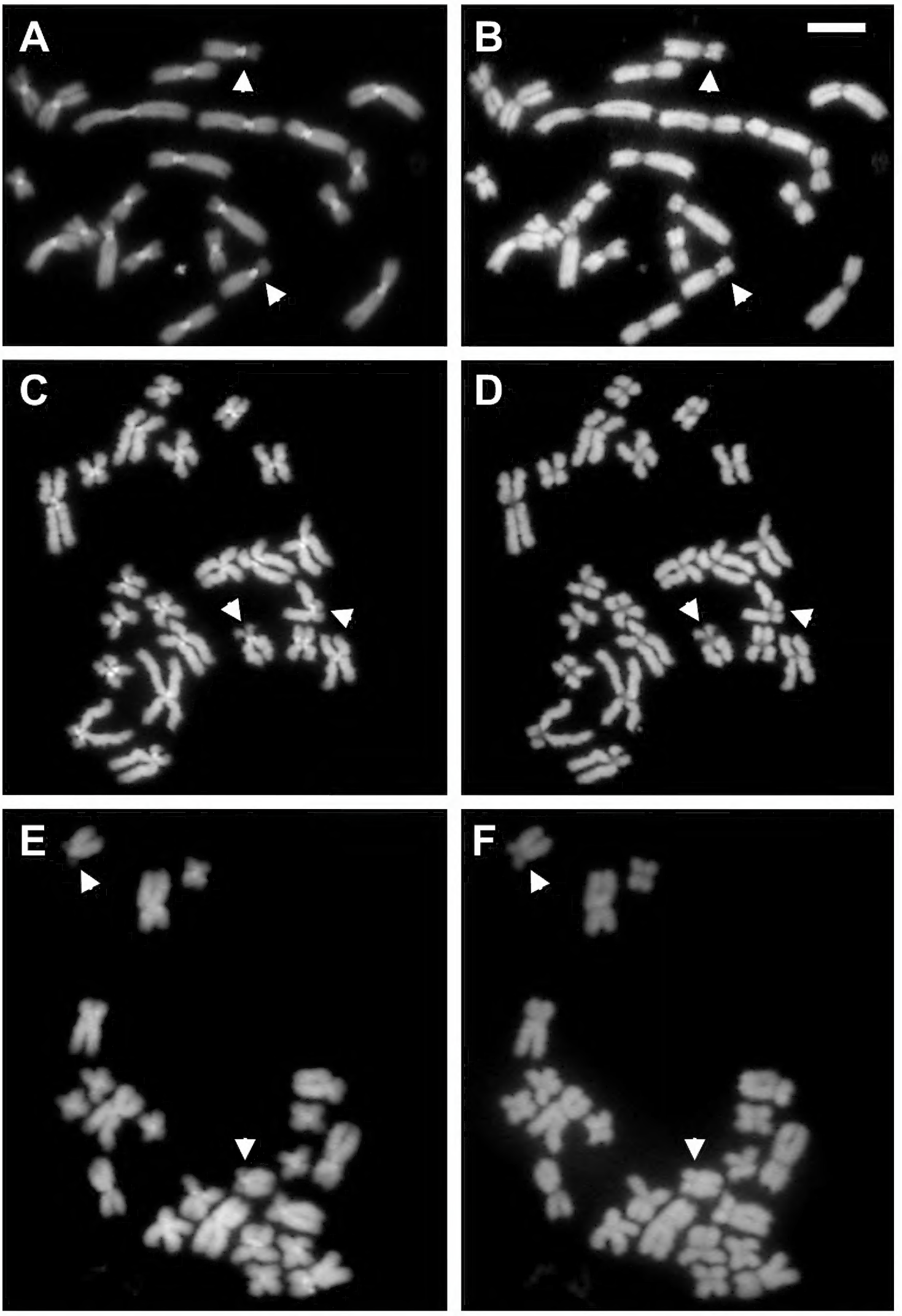

Figure 2. Mitotic metaphases of *O. barituensis* (**A, B**), *O. berdemenos* (**C, D**) and *O. discoidalis* (**E, F**) stained with DAPI (**A, C, E**) and CMA_3 (**B, D, F**), arrowheads point pairs 7. Bar = 10 μm.

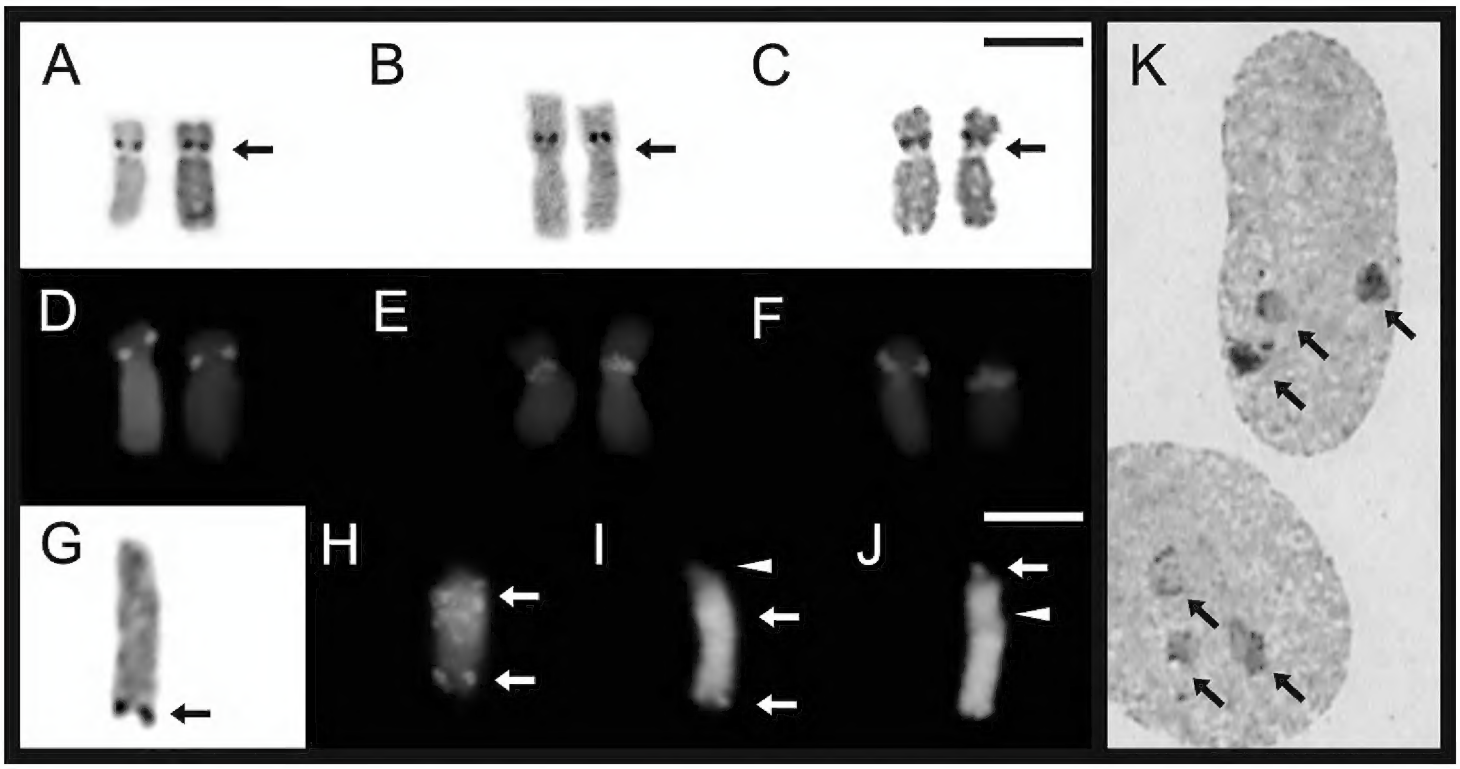

Figure 3. NORs bearing chromosome pairs (**A–C**) and rDNA (**D–F**), in *Oreobates barituensis* (**A, D**), *O. berdemenos* (**B, E**) and *O. discoidalis* (**C, F**). B_T chromosome in *O. barituensis* showing positive NORs (**G**), active and inactive rDNA (**H**), CMA_3 (**I**) and DAPI (**J**). Two interphase cells with three active NORs after silver staining (**K**). Black arrows indicate Ag-NORs, white arrows and arrowheads shows positive and negative fluorescent marks, respectively. Bars = 10 μm.

5B). These elements were always observed as an univalent, and in those individuals carrying two Bs, both were detected as univalents (Fig. 5B) or even paired as a bivalent (Fig. 5C). In the latter case, the association between both B_{st} was euchromatic rather than heterochromatic (Fig. 5C). Meiosis of these elements from individuals carrying B_d supernumeraries could not be studied due to poor quality of preparations.

Discussion

The genus *Oreobates* is composed of 23 species (Frost 2015), of which four of them were cytogenetically studied: *O. barituensis* and *O. berdemenos* (this work), *O. discoidalis* (Brum-Zorrilla and Sáez 1968, Schmid 2010, this work), and *O. crepitans* (Siqueira et al. 2009). *Oreobates* species share a similar morphology of chromosomes, as well as C-banding patterns and the location of NORs. The interstitial heterochromatic band observed in the long arm of pair 6 on specimens of the three species of *Oreobates* studied here, was not previously reported for *O. crepitans* by Siqueira et al. (2009). However, with the use of similar procedures than these authors for the C-banding protocol, the band was not detectable or was variably marked; suggesting that condensation of chromosomes may play an important role in its detection.

Within the subfamily Holoadeninae, the chromosomes of only 8 of 119 recognized species were studied (Schmid et al. 2010, and references therein). The 2n = 22 with all biarmed chromosome pairs (FN = 44) shared by *Oreobates* species are also

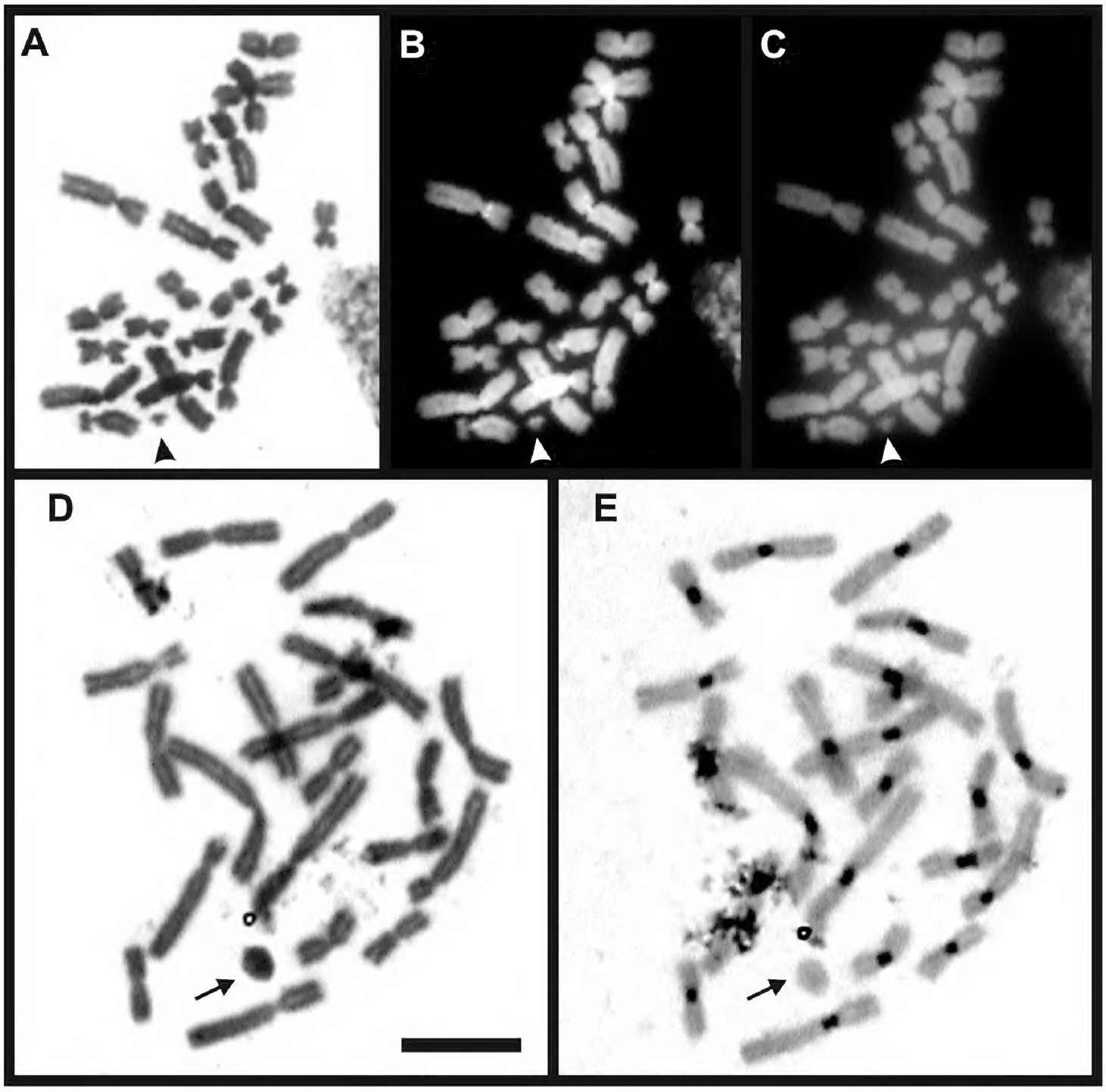

Figure 4. The small B_{st} chromosome (arrowhead) in *O. barituensis* with conventional staining (**A**), DAPI (**B**) and CMA_3 (**C**). B_d chromosome (arrow) in *O. berdemenos* evidenced after Giemsa (**D**) and C-banding (**E**). Bar = 10 μm.

present in *Phrynopus barthlenae* Lehr & Aguilar, 2002. However, *Barycholos ternetzi* (Miranda-Ribeiro, 1937) shows a 2n = 22; FN = 38, *Euparkerella brasiliensis* (Parker, 1926) 2n = 20; FN = 40, and *Holoaden bradei* Lutz, 1958 2n = 18; FN = 36 (Schmid et al. 2010). Although, *Lynchius* Hedges, Duellman & Heinicke, 2008, *Oreobates* and *Phrynopus* Peters, 1873, were recurrently recovered as related groups in several phylogenetic hypotheses (Hedges et al. 2008, Pyron and Wiens 2010, Padial et al. 2014), the scarcity of data do not allow a clear understanding of chromosome character distribution among Holoadeninae (complete absence of data for *Bryophryne*, *Hypodactylus*, *Niceforonia*, *Noblella*, and *Psychrophrynella*). However, the available karyological information suggests that a whole-biarmed karyotype of 22 chromosomes (FN = 44) is shared by species of the clade comprising *Lynchius*, *Oreobates*, and *Phrynopus*.

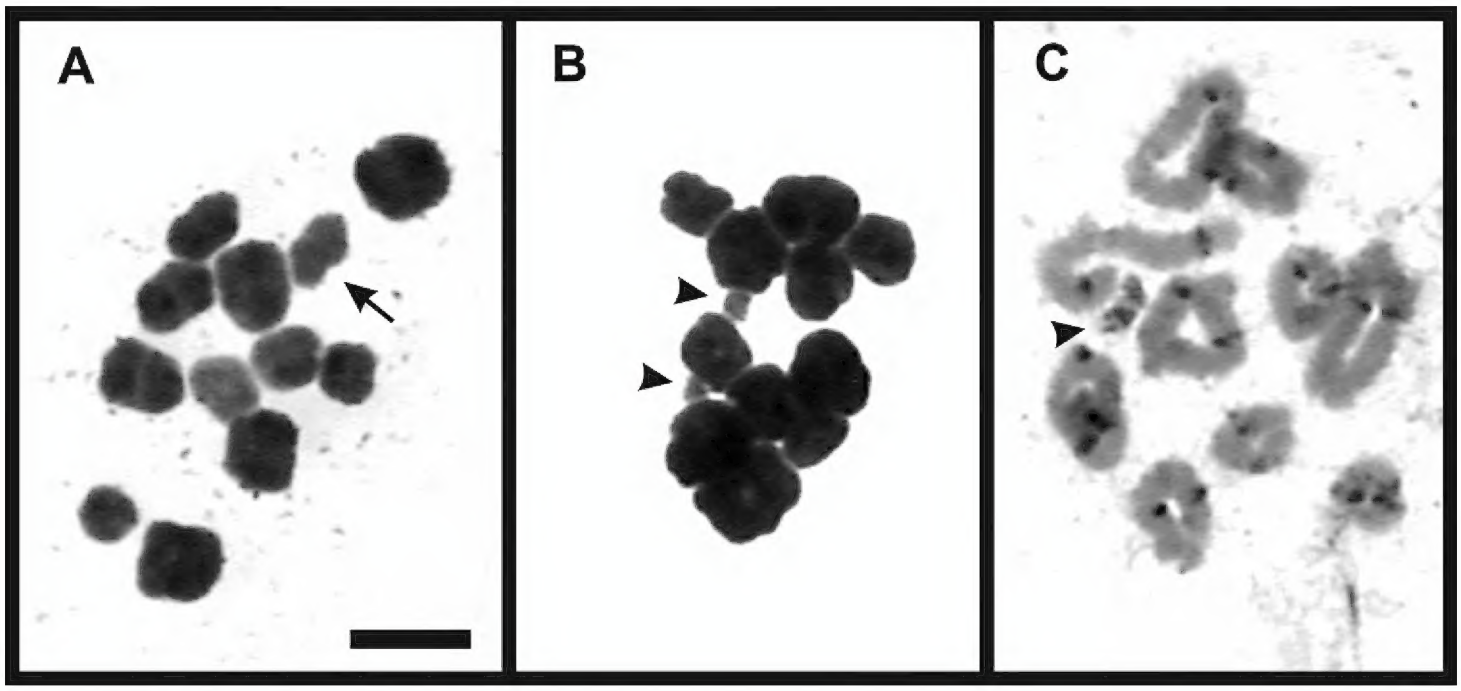

Figure 5. Meiosis I in specimens of *O. barituensis*. Metaphase I with conventional staining showing Bs as univalent: one B_T (**A**) and two B_{st} with negative pyknosis (**B**). Diakinesis after C- banding with a bivalent B_{st} (**C**). Arrow and arrowheads indicate the B_T and B_{st} respectively.

B chromosomes are widespread among eukaryotes (Beukeboom 1994, Camacho 2005), and to date have been formally reported in 20 anuran species (Green 2004 and references therein, Lanzone et al. 2008, Schmid et al. 2010 and references therein, Milani et al. 2011, Hernández-Guzmán et al. 2011, Suárez et al. 2013, Mezzasalma et al. 2015). Schmid et al. (2010) analyzed with conventional solid staining the chromosomes of 11 specimens of *O. discoidalis*, collected by J.P. Bogart in 1969 in northwestern Argentina (Aguas Negras, Jujuy). These authors found two different telocentric B chromosomes, both showing a mosaic in the chromosome number between cells of a same specimen (mitotically unstable), occurring at most once per cell. One of those Bs was smaller, similar in size to the pair 11 of the A complement, whereas the other one was larger than pair 5, resembling the B_T described in the present work for *O. barituensis*. As the specimens studied by Schmid et al (2010) were collected in late 1960s, with *Oreobates discoidalis* (= *Eleutherodactylus discoidalis*) being then the unique species recognized in Argentina, we cannot ascertain whether B_T chromosome were present in *O. barituensis* (this paper) and *O. discoidalis* (Schmid et al. 2010) or only in *O. barituensis*. However, an intriguing question is the lack of mitotic instability observed by us on this element as compared with previous reports. At present, the most accepted models for long-term evolution of B chromosomes states that the cytological behavior of Bs polymorphisms can change over time. A selfish (or parasitic) B may develop into neutral (or near-neutral) through a stabilization process induced by the standard genome of the host species (Camacho et al. 1997, Zurita et al. 1998, Camacho et al. 2000), or alternatively by regularizing their own pairing during meiosis (Araújo et al. 2001, 2002). While the mitotic instability of B chromosomes can be interpreted as a mechanism for accumulation, this phenomenon hinders an organism (or a population) to fit to an optimal number of these elements, and unless it favors the transmission of

Bs on germinal cells (i.e. by premeiotic accumulation) it would play a negative role in their long-term persistence (Nur 1963, 1969). Cavallaro et al. (2000) demonstrated that mitotic instability may take part as a possible mechanism for Bs to increase their frequency and thus invade populations. The authors observed over a ten year period a significant rise in the frequency of a B chromosome in the fish *Prochilodus lineatus* (Valenciennes, 1837). This was correlated with a decrease in the mitotic instability of the chromosome (i.e. Mitotic stabilization), suggesting that the population studied was likely under the last phase of B chromosome invasion. A possible explanation for the discrepancies observed between the present work and that of Schmid et al. (2010) concerning the mitotic behavior of the B_T in *O. barituensis* is that the instability of this element was abolished over time in the studied population (almost 50 years).

NOR-bearing B chromosomes were reported in 27 species of plants and 25 of animals [three of them anurans: *Eleutherodactylus gundlachi*, *Gastrotheca espeletia* Duellman & Hillis, 1987, and *Spea hammondii* (Baird, 1859)], showed to be supernumerary chromosomes carrying rDNA detected by silver staining and/or FISH with a rDNA probe (Green 1990, Jones 1995, Jones and Díez 2004, Silva and Yonenaga-Yassuda 2004, Camacho 2005, Cabrero and Camacho 2008, Acosta and Moscone 2010, Schmid et al. 2010, Ruiz-Estévez et al. 2013, Silva et al. 2014). The B_T chromosome in *O. barituensis* displayed active NORs in mitoses as well as in interphase nuclei, but also has interstitial heterochromatin associated with inactive 18S rDNA. A tempting target for further studies to test the origin of this B chromosome are the pair of A chromosomes carrying NORs (pair 7). It must be considered that B chromosomes may suffer a further degeneration after their origin, thus becoming heterochromatic and loosing homology with their precursors (Green 1990, Camacho et al. 2000), in addition to the observed mobility nature of rDNA sequences by transposition between non-homologous chromosomes. These facts prevent us from hypothesizing about the possible origin of this element, as B chromosomes would have acquired rDNA subsequent to their formation (Houben et al. 2013, and references therein).

Two other types of supernumerary chromosomes observed in *O. barituensis* were a small subtelocentric and heterochromatic B_{st} that occurs in a high prevalence in the locality of Tiraxi, and the euchromatic dot-like B_d, which is mitotically unstable. Interestingly, specimens of the two other species of *Oreobates* analyzed herein also showed supernumeraries B_d of similar size, and smaller than the smallest pair of the A complement. B chromosomes, which lack a functional centromere would be lost by drift (Camacho et al. 1997). The low transmission efficiency of these elements observed in somatic cells (lower than 50% per individual) due to mitotic instability would impede them to survive as true B chromosomes, unless their transmission ability would be increased in the gametes by acquiring a functional centromere. It is remarkable that the other small supernumerary elements described as B_{st} differed from those B_d by the presence of a conspicuous centromere, and because they were recorded in almost all somatic and germinal cells.

Finally, the available cytogenetic data points to the Brachycephaloidea as an extremely diverse group, with 2n ranging from 16 to 38, and FN = 26–52 (De Weese

1975, Bogart 1991, Bogart and Hedges 1995, Campos and Kasahara 2006, Green and Sessions 2007, Schmid et al. 2010). However, the occurrence of species bearing B chromosomes is not higher than in other anurans groups. Under this scenario, Schmid et al. (2010, 2012) reported the occurrence of "spontaneous somatic supernumerary marker chromosomes" as a common feature observed amongst Brachycephaloidea, at first described in the discoglossid frog *Hoplobatrachus tigerinus* (Daudin, 1802) by Yadav (1973). These elements are considered to be originated as a consequence of spontaneous chromosomal rearrangements. Like B chromosomes, are variable in composition and structure of chromatin, morphology, and behavior during mitosis, denoting thus an heterogeneous chromosome type found in more than 50 species of brachycephaloids frogs (Schmid et al. 2010, 2012). This fact leads to the issue that there might be B chromosomes or nascent Bs undetected among them, pointing to Brachycephaloidea as an interesting group for studying the origin and evolution of B chromosomes in Anurans.

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Supplementary material I

Specimens of *Oreobates* examined

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Data type: specimens data

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